

## Conformational Switching by Asparagine-Linked Glycosylation

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Received October 1, 1996

Correct folding of many glycoproteins, including the influenza virus coat protein hemagglutinin, requires asparagine-linked glycosylation.<sup>1,2</sup> The cotranslational<sup>3</sup> timing of *N*-linked glycosylation suggests that the carbohydrate may influence protein folding by altering the local secondary structure of the nascent polypeptide proximal to the glycosylation site.<sup>4,5</sup> Recent fluorescence energy transfer experiments reveal that polypeptide sequences adapted from  $\beta$ -turn regions of hemagglutinin adopt distinct structures after the covalent carbohydrate modification, suggesting that *N*-linked glycosylation triggers a change from an open conformation to a more compact conformation.<sup>6</sup> Herein we present a detailed 2D <sup>1</sup>H NMR study of the structures of the nonglycosylated (**1**) and glycosylated (**2**) peptides in water.

### 1: Ac-Orn-Ile-Thr-Pro-Asn-Gly-Thr-Trp-Ala-NH<sub>2</sub>

### 2: Ac-Orn-Ile-Thr-Pro-Asn (GlcNAc)<sub>2</sub>-Gly-Thr-Trp-Ala-NH<sub>2</sub>

The structure of these peptides is based on the A282-288 sequence of hemagglutinin.<sup>7</sup> The spectroscopic studies presented in this paper reveal that the glycosylation site of peptide **1** adopts the extended Asx-turn conformation<sup>8</sup> that is important for *N*-linked glycosylation. In contrast, glycopeptide **2**<sup>9</sup> exhibits the compact type I  $\beta$ -turn conformation observed in the final native protein structure.

Analysis of ROESY spectra and amide proton variable temperature (VT) coefficients<sup>10</sup> reveal different peptide backbone conformations at the glycosylation sites of peptides **1** and **2**. The NMR data for peptide **1** were consistent with an Asx-

turn conformation. In this peptide, an Asx-turn conformation would be characterized by hydrogen-bonding between the threonine amide proton and the carboxamide carbonyl of the asparagine side chain.<sup>11</sup> NOEs indicative of an Asx-turn are present in the ROESY spectrum (Figure 1A and Table 1), in particular  $d_{\text{NN}}(\text{Gly}^6, \text{Thr}^7)$ . Furthermore, the low VT coefficient observed for the Thr<sup>7</sup> amide proton (Table 2) is also characteristic of the hydrogen bond found in an Asx-turn. Taken together, these data support an Asx-turn for this peptide.

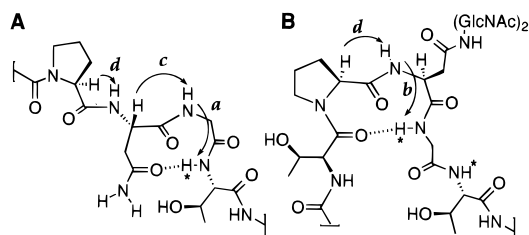
Distinct differences between the ROESY spectra of glycopeptide **2** and peptide **1** were observed, indicating a change in the conformation upon glycosylation. The diagnostic  $d_{\text{NN}}(\text{Gly}^6, \text{Thr}^7)$  NOE crosspeak, indicative of an Asx-turn in nonglycosylated peptide **1**, was not observed for peptide **2**. Furthermore, the intensity of the  $d_{\text{NN}}(\text{Asn}^5, \text{Gly}^6)$  crosspeak was considerably stronger and a substantial decrease in the glycine amide VT coefficient was observed for peptide **2**. The Gly<sup>6</sup> amide proton, while solvent exposed in an Asx-turn conformation, is expected to be protected by the *i* to *i*+3 [CO to NH] hydrogen bond characteristic of a  $\beta$ -turn (Table 2). Thus, both the variations in NOE intensities and amide proton VT coefficients are consistent with a change from an Asx-turn to a  $\beta$ -turn upon glycosylation (arrows *a*, *b*, and *c* in Figure 1). Although the Thr<sup>7</sup> amide proton VT coefficient remained low for peptide **2**, the observed NOEs are inconsistent with an Asx-turn. A possible explanation for this low VT coefficient is suggested by the observation of an unusual and strong  $d_{\text{N}\alpha}(\text{Gly}^6, \text{Thr}^7)$  NOE which indicates that the threonine may fold back, thereby offering some protection of the threonine amide which is unrelated to the  $\beta$ -turn (Figure 2B).

Although the NMR data indicated the presence of a  $\beta$ -turn in the glycosylated peptide, it was difficult to distinguish between the topologically similar type I and type II  $\beta$ -turns using NOE data alone due to similar interproton distances expected in the two structures.<sup>12</sup> This problem is compounded by the presence of a proline residue, which lacks an amide proton, therefore removing the possibility of observing additional, diagnostic NOEs. However, ideal  $\phi$  (N-C $\alpha$ ) dihedral angles for the residue at position *i*+2 in the type I and type II  $\beta$ -turns are very distinct, being  $-90^\circ$  and  $90^\circ$ , respectively.<sup>12</sup> A high <sup>3</sup> $J_{\text{HN}\alpha}$  coupling constant should be noted for the type I structure;<sup>13</sup> a type I  $\beta$ -turn structure is expected to have an asparagine coupling constant of approximately 8 Hz, while the type II turn would exhibit a value closer to 5 Hz. The <sup>3</sup> $J_{\text{HN}\alpha}$  coupling constant for asparagine in **2** was 9.6 Hz, a value falling well within the range of the type I  $\phi$ -angle as predicted by the modified Karplus curve. Additionally, two contiguous L-amino acids (at *i*+1 and *i*+2) have a greater propensity to form a type I turn, since a type II turn requires adoption of backbone dihedral angles that are energetically unfavorable for L-amino acids.<sup>14</sup>

The solution state structures of peptides **1** and **2** were solved by a simulated annealing procedure,<sup>15</sup> (also see Supporting Information) revealing that both peptides are well ordered in the central region of the sequence that includes the glycosylation site (Figure 2). For peptide **1**,  $\phi$  and  $\chi_1$  dihedral restraints for threonine calculated from vicinal coupling constants, along with 96 NOE-derived distances obtained from the ROESY experiments, were included in the simulated annealing protocol. While the simulated annealing experiments using only these data generated a well-defined backbone at the glycosylation site, the asparagine side chain was poorly defined which is partially due

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- (9) Glycopeptide **2** was prepared by chemical synthesis from a peptide in which allyl-protected aspartic acid was substituted for asparagine. While on the solid support, the allyl ester was removed with tetrakis(triphenylphosphine)palladium, and chitobiosylamine was coupled to the side chain with BOP activation. (Cohen-Anisfeld, S. T.; Lansbury, P. T. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537). The Hmb (2-hydroxy-4-methoxybenzyl) amide-protected glycine was utilized to prevent succinimide formation (Offer, J.; Quibell, M.; Johnson, T. *J. Chem. Soc., Perkin Trans. 1* **1996**, 175–182). Efficient Hmb cleavage after glycan coupling was ensured by treating the resin-bound peptide with hydrazine hydrate (5% v/v) to deacetylate the 2-hydroxy group. Predicted masses of peptide **1** (1015) and glycopeptide **2** (1422) were confirmed by electrospray mass spectrometry.
- (10) NMR data were acquired at 7 °C in 90:10 H<sub>2</sub>O/D<sub>2</sub>O. ROESY spectra were acquired on a 600 MHz Varian Unity Plus spectrometer with a mixing time of 400 ms using presaturation to suppress the water signal. VT data were calculated from TOCSY spectra acquired on a 500 MHz Bruker spectrometer at temperatures ranging from 7 to 27 °C. Dihedral angle ( $\phi$ ) restraints were calculated from <sup>3</sup> $J_{\text{HN}\alpha}$  coupling constants obtained from double quantum filtered COSY experiments acquired at 7 and 25 °C. Twice the number of data points (4096) were acquired for enhanced resolution. All <sup>3</sup> $J_{\text{HN}\alpha}$  values used to calculate dihedrals were within the reliable range<sup>12</sup> (no value below 9.6 Hz was used in the structure calculation). Side chain ( $\chi_1$ ) dihedral angles were calculated from  $\alpha$ - $\beta$  coupling constants from spectra taken at 25 °C. (Pachler, K. G. R. *Spectrochim. Acta* **1964**, *20*, 581–587).

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**Figure 1.** A. Structure of Asx-turn. B. Structure of Type I  $\beta$ -turn. The Gly amide Thr (from  $d_{NN}(\text{Gly}, \text{Thr})$ ) interproton distance  $a$  is expected to be shorter in an Asx-turn. The Asn amide Gly amide [from  $d_{NN}(\text{Asn}, \text{Gly})$ ] interproton distance  $b$  is expected to be shorter for a  $\beta$ -turn. The amide protons indicated by the asterisks show reduced amide proton VT coefficients.

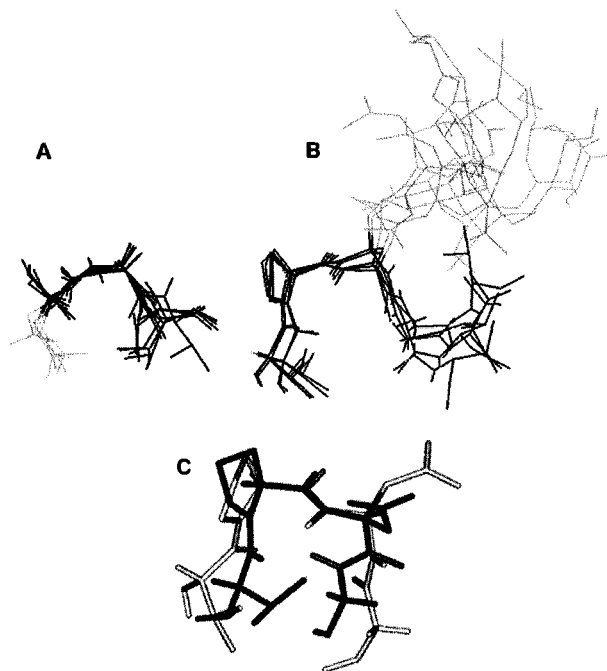
**Table 1.** Relative Intensities of Diagnostic ROESY Crosspeaks in the Turn Regions of Peptides 1 and 2

	NOE	1	2
$a$	$d_{NN}(\text{Gly}^6, \text{Thr}^7)$	weak	absent
$b$	$d_{NN}(\text{Asn}^5, \text{Gly}^6)$	weak	strong
$c$	$d_{\alpha N}(\text{Asn}^5, \text{Gly}^6)$	strong	medium
$d$	$d_{\alpha N}(\text{Pro}^4, \text{Asn}^5)$	strong	strong

**Table 2.** Amide Proton Variable Temperature Coefficients ( $-\Delta\delta/\Delta T$ , ppb/K) of Peptides 1 and 2<sup>a</sup>

residue	1	2
Thr	10.2	9.8
Asn	7.0	8.6
Gly	7.8	6.8
Thr	6.2	6.0

<sup>a</sup> Data points were taken at 5° increments from 280 to 300 K. (See Supporting Information for experimental data.)



**Figure 2.** Backbone superimposition of simulated annealing structures. A. Overlay of 7/10 structures derived from a simulated annealing procedure for peptide 1. Residues Asn<sup>5</sup> to Thr<sup>7</sup> are shown. The asparagine side chain is highlighted in gray. B. Overlay of 5/10 structures for peptide 2. Residues Thr<sup>3</sup> to Thr<sup>7</sup> are shown, with the asparagine-linked saccharide highlighted in gray. C. Superimposition of the backbone of the A284-287 sequence taken from the hemagglutinin crystal structure<sup>1b</sup> (in black) with a low-energy structure of glycopeptide 2 (in gray).

to the small number of protons on the side chain. Therefore, it was desirable to also determine the conformation of the asparagine side chain. There are three sterically favored side chain conformations: gauche (+), gauche (−), and trans. A gauche (+) conformation was ruled out by the unequal intensities found for the two Asn  $\beta$  protons in the  $\alpha$ - $\beta$  ROESY crosspeak. The Asn  $\chi_1$  restraint was therefore simultaneously

constrained to the trans and the gauche (−) conformation in a subsequent simulated annealing procedure. When this constraint was applied, 9/10 structures generated converged to the trans side chain rotamer. The backbone and Asn  $\chi_1$  dihedrals for 7 of the 10 structures were similar to the I<sub>t</sub> Asx-turn model proposed by Abbadi *et al.*<sup>11</sup>

One hundred nine NOE-derived distance restraints, 22 of which involved the saccharide, and the asparagine  $\phi$  dihedral restraint range (−150° to −80°) were included in the simulated annealing procedure implemented for glycopeptide 2. From this analysis, 5/10 structures exhibited dihedral angles typical of a type I  $\beta$ -turn. The remaining structures had aberrant proline  $\psi$ -angles which was both unsurprising and unavoidable, given the lack of NOE data for proline. The strong NOE  $d_{\alpha N}(\text{Pro}^4, \text{Asn}^5)$  was assigned a restraint of 1–5 Å and resulted in a distance of 3.6 Å for all structures. This distance is consistent with a type I turn (the corresponding distance in a type II turn would be 2.2 Å); additionally, the dihedral angles are close to those predicted for a type I turn (see Supporting Information).

The NMR analysis of glycopeptide 2 reveals no significant evidence of specific interactions between the peptide and the carbohydrate. Only three very weak NOE interactions between the carbohydrate and the side chain of Trp<sup>8</sup> were observed. These weak NOEs may be due to the large relative size of the indole moiety and are unlikely to reflect a specific peptide carbohydrate interaction. Notably, isoleucine is found at this position in the native hemagglutinin sequence. Therefore, it is likely that the conformational change observed for the glycopeptide results from either a steric effect in which the carbohydrate alters the conformational space available to the peptide or from a modulation of the local water structure that influences the environment that the peptide experiences.

This NMR analysis demonstrates that asparagine-linked glycosylation induces a change in the backbone conformation of a small peptide in aqueous solution. Whereas the nonglycosylated peptide adopts an Asx-turn conformation, the  $\beta$ -turn conformation favored after glycosylation is similar to that found in the final folded native protein hemagglutinin (Figure 2C).<sup>16</sup> In fact, glycosylation sites are frequently found at  $\beta$ -turns in native proteins.<sup>17</sup> Thus, the cotranslational carbohydrate modification may alter the conformational space available to the nascent peptide such that the adoption of a small secondary structural element is favored which may facilitate subsequent folding events.

**Acknowledgment.** This work was supported by the NIH (GM39334). S.E.O. acknowledges an NIH Predoctoral Biotechnology Training Grant (GM08346). We also gratefully acknowledge the Dorothy Chandler, Camilla Chandler Frost Laboratory in Biology at Caltech for use of the Varian Unity Plus 600 MHz NMR Spectrometer.

**Supporting Information Available:** Amide proton variable temperature data, NOE restraints, description of the simulated annealing procedure, and dihedral angles of the generated structures (9 pages). See any current masthead page for ordering information and Internet access instructions.

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(15) Simulated annealing (SA) was performed using Biosym's NMRchitect. The number of restraints given is before the pseudoatom correction. ROESY crosspeaks were sorted into weak (2–4 Å) and strong (1–3 Å) bins on the basis of visual assessment of relative crosspeak intensities. A few very weak long-range NOEs were included in the annealing restraint file (Supporting Information). Peptide 1 had five long-range NOEs (four involving the tryptophan side chain) and peptide 2 had four (three involving the tryptophan side chain). The hydrogen bond suggested by the VT data was included as a distance restraint (2–5 Å) in the SA for peptide 1. Since the variable temperature analysis of 2 revealed two relatively low VT coefficients, NOE and coupling constant data were used exclusively in the SA database and no assumptions were made regarding the hydrogen-bonding network in the glycopeptide. All dihedral restraints were assigned a 70° or 80° range. Root mean square deviation (rmsd) values were calculated from an average molecule for carbon and nitrogen atoms. The high rmsd value for Asn<sup>5</sup> in peptide 2 reflects the disorder of the chitobiose moiety. Peptide 1 (in Å): Asn<sup>5</sup> 0.80 ± 0.14; Gly<sup>6</sup> 0.40 ± 0.08; Thr<sup>7</sup> 0.75 ± 0.27. Peptide 2 (in Å): Thr<sup>3</sup> 0.93 ± 0.25; Pro<sup>4</sup> 0.50 ± 0.11; Asn<sup>5</sup> 3.9 ± 0.90; Gly<sup>6</sup> 0.61 ± 0.05; Thr<sup>7</sup> 1.3 ± 0.61.

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